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Morphological and Anatomical Variation in Sporophylls of *Isoetes sinensis* Palmer (Isoetaceae), an Endangered Quillwort in China

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Abstract.—*Isoetes sinensis* is a rare and endangered plant in wetlands of southeastern China. Previous studies have reported the chromosome number, geographic distribution, and ecology of this Asian endemic, but there has not been an analysis of diagnostic characters associated with the sporophylls of *I. sinensis* in China. Therefore, morphological and anatomical variation of sporophylls in the three known Chinese populations of *I. sinensis* were evaluated and compared. The variation found is discussed in relation to the present taxonomy of *I. sinensis*.

Isoetes L. is a cosmopolitan genus with an estimated 150 extant species (Tryon and Tryon, 1982; Talyor and Hickey, 1992). Five species of *Isoetes* are known to occur in China. These include three basic diploids ($2n = 22$): *I. yunguiensis* Wang Q. F. & W. C. Taylor, *I. hypsophila* Handel-Mazzetti and *I. taiwanensis* DeVol, one tetraploid ($2n = 44$) *I. sinensis* Palmer, and one hexaploid ($2n = 66$) *I. orientalis* H. Liu & Q. F. Wang (Liu *et al.*, 2002; Wang *et al.*, 2002; Liu *et al.*, 2005). Palmer's (1927) original description of *Isoetes sinensis* was based on specimens from Nanjing, China, from which he described the megaspores as "thickly set with high, stout, blunt or crested columns, more or less confluent into crested ridges especially below" and the microspores as "densely spinulose." *I. sinensis* has been reported from China, Japan, and Korea (Takamiya *et al.*, 1997; Takamiya, 2001). The tetraploid cytotype of *I. sinensis* was first reported in China by He *et al.* (2002). A hexaploid taxon, found in Korea and Japan, was originally described as *I. coreana* by Chung and Choi (1986), but Takamiya *et al.* (1997) treated this cytotype as *Isoetes sinensis* var. *coreana* (Y. H. Chung & H. K. Choi) M Takamiya, M. Watanabe & K. Ono.

Isoetes sinensis is an aquatic fern confined to clear waters (Pang *et al.*, 2003). In recent years, it has decreased in numbers and even disappeared from several locations in mainland China (Foster *et al.*, 1974). In our recent field investigation, only three populations remained in mainland China. It is now considered to be rare and endangered in China and it is listed as a first category protected wild plant species (Yu, 1999).

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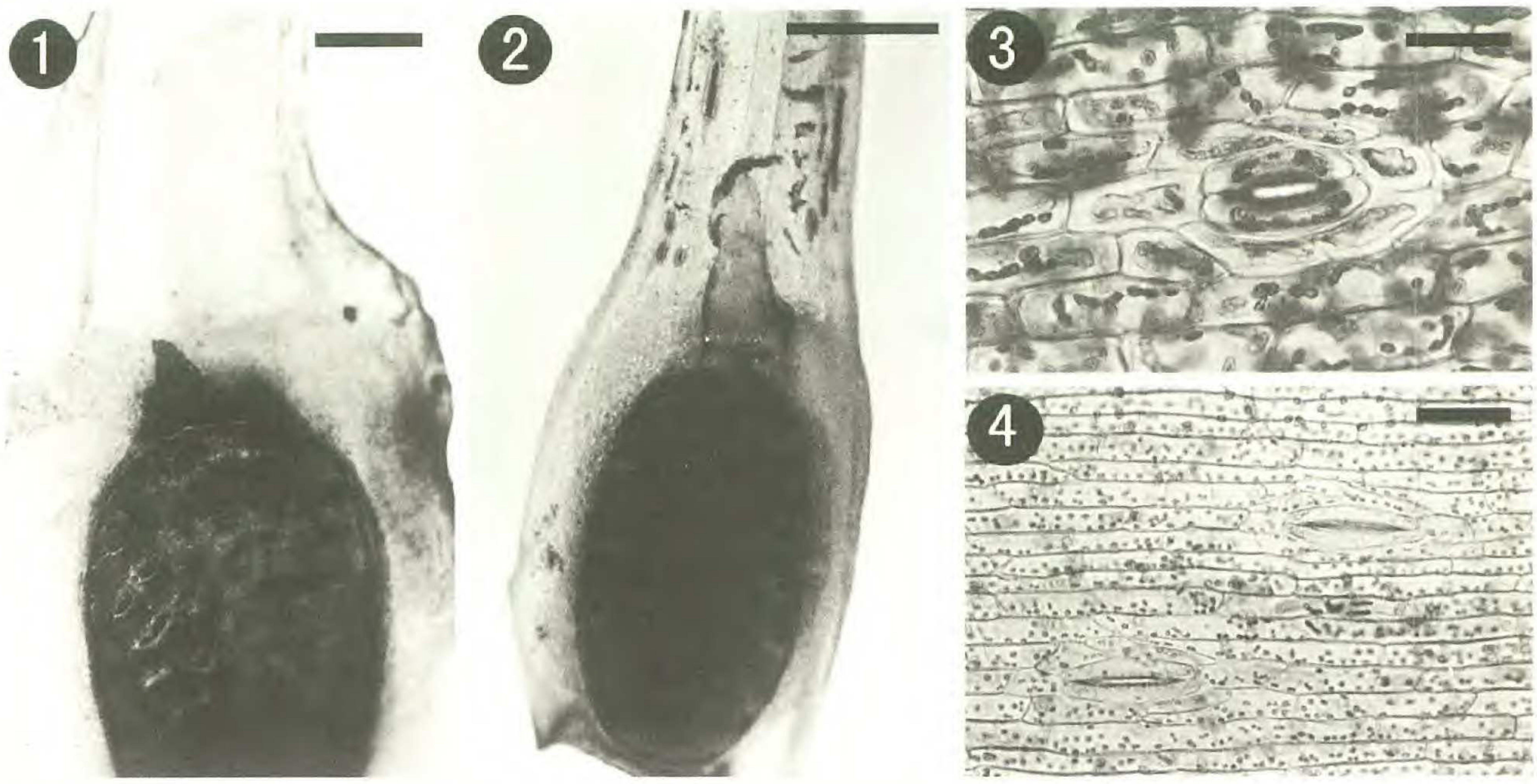


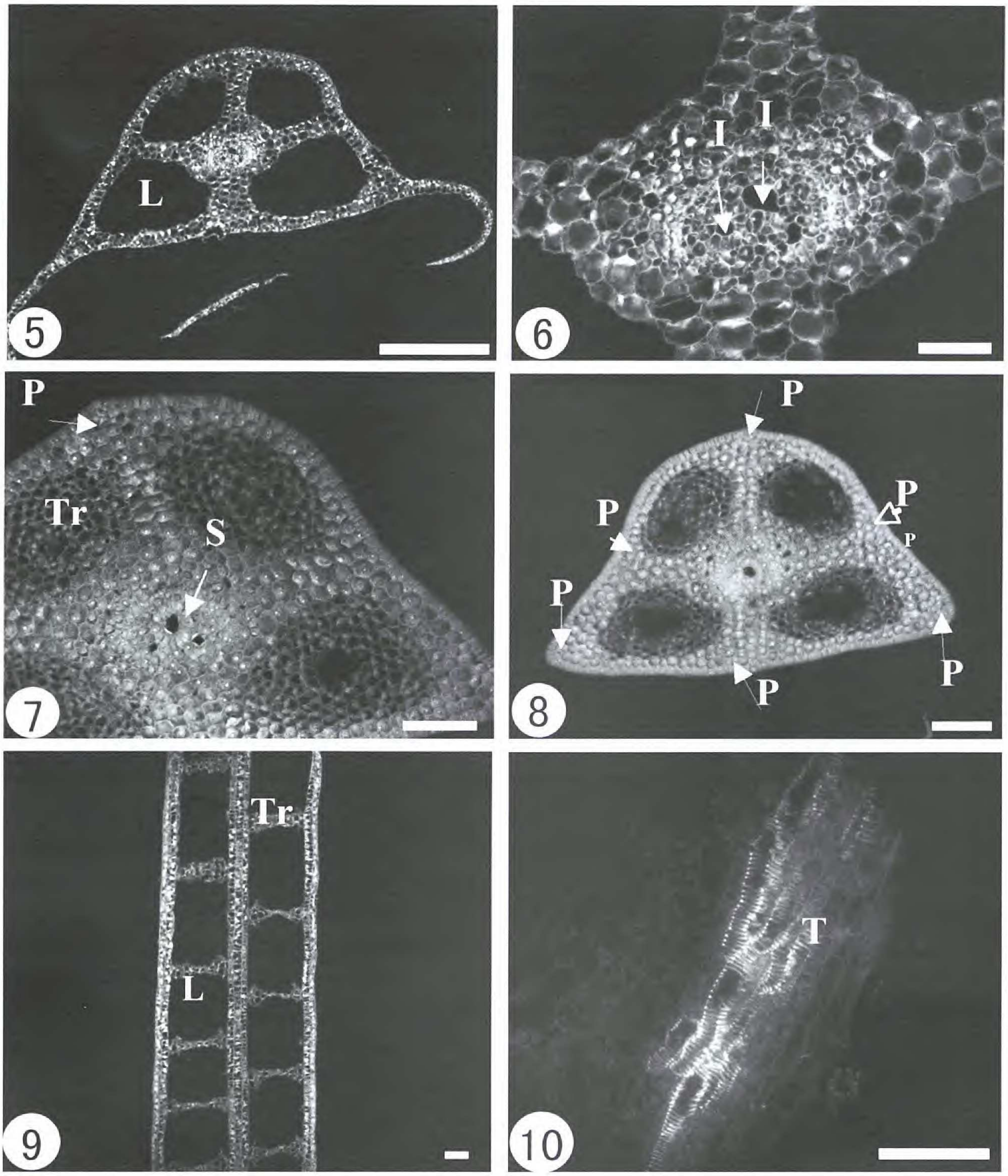
FIGS. 1–4. Foliar characters of *I. sinensis*. 1. Leaf base of megasporophyll showing small, deltate ligule (pop. JD 1). 2. Leaf base of megasporophyll showing long triangular ligule (pop. XN 1). 3. Close up of epidermis and stomatal apparatus (pop. JD 1). 4. Epidermis and stomatal apparatus (pop. XL 1). Bars in 1, 2 = 4 mm; bars in 3, 4 = 40 μm.

Plants of *I. sinensis* have linear leaves arising spirally from a depressed shoot apex on an underground, subglobose, 3 or 4-lobed rootstock. Its dichotomous roots arise synchronously from the furrows between the lobes on the undersurface of the rootstock. Leaves of *I. sinensis* are brittle, stiffly erect, and each contains four longitudinal air canals (Figs. 5–8). The length of mature leaves ranges from 15–30 cm. Sporangia develop in a concavity in the expanded base of each leaf (Figs. 1, 2). A deltoid ligule occurs above the concavity. Plants are typically monoecious with external megasporophylls and internal microsporophylls; dioecious plants are rare. Megasporphylls are produced earlier than microsporophylls in the growing season, are peripherial and have megasporangia containing 200–500 globose megaspores averaging about 450 μm in diameter. Microsporophylls are produced later in the growing season are more central and each has a microsporangium containing thousands of ellipsoid microspores averaging about 30 μm in length.

The goals of this study were to document morphological and anatomical variation in sporophylls across and within three populations of *I. sinensis* in China and evaluate how this variation relates to the present taxonomy of *I. sinensis*.

MATERIALS AND METHODS

Three populations of *Isoetes sinensis* were identified in southeastern China. Population XN1 lies in a marshy and abandoned field at Xiuning, Anhui Province at an elevation of 360 m. Populations JD1 and JD2 are in Jiande, Zhejiang Province at an elevation of 134 m. The latter two populations occur



FIGS. 5–10. Anatomical characters of *I. sinensis*. 5. Proximal leaf transverse section showing four air chambers (pop. JD 1). 6. Close up of Fig 3 showing three intrastelar canals (pop. JD 1). 7. Distal leaf transverse section showing three intrastelar canals and leaf trabeculae (pop. JD 2). 8. Distal leaf transverse section showing four air chambers and one intrastelar canal (pop. XN 1). 9. Longitudinal leaf section showing air chambers and diaphragms, uniform within populations JD 1, JD 2 and XN 1. 10. Longitudinal section showing annular tracheids. Bar in 5 = 800 µm; Bars in 6–9 = 200 µm; Bar in 10 = 75 µm; I = intrastelar canal; L = lacuna; P = peripheral fiber strand; S = stele; T = tracheid; Tr = trabeculae.

in the littoral zone of Xingan-jiang River where, due to an upstream hydroelectric dam, water level changes diurnally to completely expose plants for several hours each day. JD1 was separated from JD2 by a one kilometer stretch of coast along the river. Mature plants were collected from each of the three populations in December, 2002. Each plant was labeled and cultivated in a shallow pot in the Wuhan University Botanical Garden.

Morphological features of sporophylls, including number and length of sporophylls, ligule shape, velum, megaspore number per megasporangium, spore size, and guard cell length, were recorded from fresh, field-collected specimens and from specimens fixed in FAA. Anatomical features of sporophylls were observed in cultivated plants from hand sections of fresh samples and microtome sections of fixed samples. A Leica DMIRE2 microscope fitted with a MicroMax CCD Camera employing Meta-Morph software (Universal Imaging Corporation, series 4.0) were used to capture images. Microscopic measurements were made using an ocular micrometer.

Sporophyll number was counted and sporophyll length was measured for twenty-five plants randomly selected from each of the three populations. Ligule shape was evaluated on each of these sporophylls. To gauge stomata guard cell length, the leaf epidermis was peeled from five plants in each population and the length of twenty-five guard cells from each plant was measured. To establish the average number of megaspores per megasporangium, the number of megaspores in 200 megasporangia from each population, were counted. For spore size, one mature megasporangium and microsporangium were taken from each of five randomly chosen individuals in each population. Twenty air dried megaspores and twenty re-hydrated microspores were measured from each sporangium for a total sample size of 100 megaspores and 100 microspores from each population. Leaf sections were made just above the insertion of the ligule and at the mid-length of the leaf.

Specimens for microtome sectioning were harvested, fixed in FAA, and after passing through an alcohol dehydration series were embedded in paraffin (Johanson, 1940). Microtome sections were cut at 6–8 μm.

Results and Discussion

Morphological features.—Scale leaves were absent and the velum was rudimentary in all the plants sampled from all three populations. The labium in populations JD1 and JD2 was larger than in population XN1. The labium of plants in populations JD1 and JD2 was ca. 680 μm long, whereas the labium in population XN1 was slightly shorter, ca. 600 μm long. The labium width in JD1 and JD2 was ca. 300 μm, whereas labium width in population XN1 was considerably smaller, ca. 130 μm wide. Ligule shape also showed variation. Plants of XN1 had ligules that were long triangular and prominent, but in JD1 and JD2 the ligule was deltate, cordate, shorter, and indistinct (Table 1). Representative ligule shapes of *I. sinensis* from populations JD1 and JD2 are shown in Fig. 1 and from population XN1 in Fig. 2.

TABLE 1. Comparison of different populations *Isoetes sinensis.*

Characters	Population		
	XN1	JD1	JD2
Location	Xiuning, Anhui	Jiande, Zhejiang	Jiande, Zhejiang
Chromosome number	44	44	44
Elevation (m)	360	134	134
Reproduction	sexual	sexual	sexual
Habitat	abandoned rice field (marsh)	fresh water intertidal zone	fresh water intertidal zone
Habit	emergent	submerged	submerged
Scale leaf	absent	absent	absent
Labium	present, tumefy	present, subulate	present, subulate
Velum	absent	absent	absent
Ligule shape	elongated and deltoid	cordate and smallish	cordate and smallish
Corm lobe number	3	3–4	3–4
Leaf number (n = 25)	12.08 ± 7.53	44.60 ± 34.12	13.2 ± 7.46
Leaf length (cm, n = 25)	13.08 ± 4.91	12.54 ± 3.98	7.96 ± 3.32
Peripheral fibres	4	4–6	4
Intrastelar canal number	1	3	3
Guard cell length (μm)	63.1 ± 7.28	65.42 ± 4.76	67.35 ± 5.70
Megaspore number (n = 200)	235.2 ± 87.26	411.6 ± 115.45	397.8 ± 166.28
Megaspore ornamentation	cristate	cristate	cristate
Megasopre diameter (μm; n = 100)	448.8 ± 43.91	418.9 ± 31.36	354.3 ± 36.68
Microspore ornamentation	echinate	echinate	echinate
Microspore length (μm; n = 100)	27.13 ± 2.71	34.07 ± 3.21	27.63 ± 3.24

In all three populations investigated, the leaves of *I. sinensis* were light green and erect to stoutly ascending. There was notable variation in leaf length, leaf number and spore size among populations (Table 1). The mean leaf lengths in the three populations were 13.08 ± 4.91 cm (XN1, n = 25), 12.54 ± 3.98 cm (JD1, n = 25), and 7.96 ± 3.32 cm (JD2, n = 25). The mean numbers of leaves per plant in the three populations were 12.08 ± 7.53 (XN1, n = 25), 44.60 ± 34.12 (JD1, n = 25), and 13.2 ± 7.46 (JD2, n = 25). The mean number of megaspores per megasporangium (n = 200) of JD2 is about 411.6, much larger than the 235.2 mean of XN1. Average megaspore size of XN1 is 448.9 ± 43.91 μm, much larger than that of JD2 (354.3 ± 26.67 μm). The average microspore size of JD1 is the largest among the three populations. The size of megaspores and microspores produced by *I. sinensis* is variable and appears to be dependent upon climate and locality. Love (1962) reported that the smallest spores of boreal aquatic *I. echinospora* were found on plants from the deeper and colder parts of lakes, whereas the spores were invariably larger in shallower and warmer parts of the same lakes. These observations also indicate that local environments can affect *Isoetes* spore size. Cox and Hickey (1984) stated that "Collections of a single species along a transect from shallow to deeper water show a corresponding increase in leaf length" and their analysis of three populations of *I. storkii* found that the population "highest in altitude has the smallest spores". The data from three populations of *I. sinensis* seem fully supports the first statement of Cox and Hickey (1984), but differ from their latter view.

Pfeiffer (1922) considered the presence or absence of stomata to be a reliable taxonomic character in *Isoetes*, whereas Kott and Britton (1985) considered it to be a feature dependent upon growing conditions. The presence of stomata increases photosynthetic potential and safeguards xylem from cavitation (Woodward, 1998). Numerous stomata were observed on the upper one-third of the leaves in all three populations of *I. sinensis* examined. Stomatal guard cell length showed slight variation among the three populations investigated. The guard cell length for JD1 was 65.42 ± 4.76 µm, JD2 was 67.35 ± 5.70 µm and for XN1 was 63.1 ± 7.28 µm.

Anatomical features.—Leaf shape in transverse section at the uppermost part of the membranous leaf margin reveals features common to all *Isoetes*. For example, in Figs. 5 and 8, the four air chambers surrounding the stele can be seen; trabeculae or cross partitions composed of parenchyma (Figs. 7, 9) also characterize the leaves of *Isoetes*.

Within the stele, three small cavities called intrastelar canals were present in JD1 (Figs. 5–6) and JD2 populations (Fig. 7), but only one intrastelar canal was seen in XN1 (Fig. 5). Previous studies revealed that the number of canals appeared to be a stable diagnostic feature for *Isoetes* (Takamiya *et al.*, 1997). Around the intrastelar canal, the annular tracheids can be seen clearly (Fig. 10). The internal leaf anatomy of *Isoetes* shows less environmentally induced variation than the external leaf morphology. Therefore, because of their stability, anatomical characters of leaves have been considered to be of greater diagnostic value (Pfeiffer, 1922).

The peripheral fiber strands (long arrows in Figs. 7 and 8) in *Isoetes* leaves are bundles of thick-walled fibers situated under the epidermis that provide structural support. The presence or absence and the number and position of peripheral fiber strands have been used as diagnostic characters by Pfeiffer (1922) and Wanntorp (1970). West and Takeda (1915) examined the leaf anatomy of *Isoetes* in Japan and found that the peripheral fiber strands were located in four main (one abaxial and three adaxial) bundles and two lateral accessory bundles that were not always continuous along the length of leaves. They also noted that the presence or absence of these strands in the leaves appeared to depend upon the environmental conditions under which the plants grew. In the three populations examined for this study, the peripheral fiber strands were always seen at the four main sites (long arrows in Fig. 8) and the two lateral accessory sites (short arrows in Fig. 8) and the lateral strands were always discontinuous.

Taxonomic Treatment.—Isoetes sinensis, described from China by Palmer (1927), also occurs in Japan and Korea. Takamiya *et al.* (1994) recognized $2n$ = 44, 65, 66, and 68 cytotypes for *I. sinensis*. Later, Takamiya *et al.* (1997) revised this earlier taxonomy recognizing the tetraploid cytotype as *I. sinensis* var. *sinensis* and the $2n$ = 65, 66, and 68 cytotypes as *I. sinensis* var. *coreana* (Y. H. Chung & H. K. Choi.) M. Takamiya, Mitsu. Wantanabe & K. Ono. In addition to chromosome number, there are differences in guard cell length, spore size, and leaf width between the tetraploid and hexaploid cytotypes. Takamiya *et al.* (1997) reported a significant difference ($p < 0.001$) in guard cell length between the tetraploid (mean = 65.2 μm) and the hexaploid (mean = 92.7 μm) cytotypes. Sporangium size in *I. sinensis* var. *sinensis* is generally smaller than in var. *coreana*. Megaspore diameter of the cytotypes ranged from 363 to 509 μm (mean = 435.1 μm) and from 375 to 538 μm (mean = 447.1 μm). The difference in leaf width can be seen in Takamiya *et al.* (1997, Table 1). Watanabe *et al.* (1996) reported that the mean microspore length of the tetraploid was 27.6 μm, whereas in the hexaploid it was 31.9 μm. After comparison of the spore and leaf morpholgy of *I. sinensis*, it appears that only the tetraploid cytotype of *I. sinensis* var. *sinensis* has been found in China.

Isoetes sinensis is extremely rare and endangered and is vulnerable due to its restricted range. Only the three small populations evaluated here are known to persist in China. Recent field studies have revealed that several previously reported populations no longer exist. This species is potentially threatened by any activities that would affect the habitat stability in the various populations. *Isoetes sinensis* can only survive if suitable habitat is preserved. At present, none of these populations occur in protected areas. Habitat modification, such as dumping sand to create beaches, pollution, and increasing urbanization in China continue to raise the risk of extinction of *I. sinensis*. More information needs to be gathered on the species' biology and habitat requirements in order to design and implement effective protection plans for saving this rare plant from extinction.

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